

Purification and Characterization of the Lipase of *Pseudomonas fragi*

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SUMMARY

The lipase of *Pseudomonas fragi* (NRRL B-25) was purified 75- to 100-fold with an overall recovery of ca. 20 %.

The enzyme was found to exist in heavy and light forms exhibiting the same position specificity for triglycerides, Michaelis constants (K_m), and apparent pH and temperature optima. The light form of the enzyme appeared to be present in the heavy one, probably in a complexed state.

The purified lipase was found to hydrolyze only glycerol esters of fatty acids; it required a water-fat interface and exhibited a 1,3-position specificity for triglycerides. The optimum pH for the purified enzyme with purified tributyrin as substrate was calculated to be 8.6 to 8.7 from initial velocity measurements with a pH-stat at 25°. The K_m for the purified lipase with tributyrin was found to be 0.9×10^{-3} M at 25° and pH 7.2. Exposure of the purified enzyme to 40° for 10 min. caused a complete loss of activity. A 50 % loss of activity occurred after 10 min. exposure to about 35°. When exposed to pH values ranging from 5.3 to 9.5 for 1 hr at 2° and then assayed at pH 7.0 and 35°, the purified enzyme was found to be stable in the pH range 6.6-7.8.

INTRODUCTION

Although the existence of lipolytic bacteria has been recognized for many years, our understanding of bacterial lipolysis stems for the most part from studies with crude enzyme systems. Apart from the studies with a partially purified lipase (glycerol ester hydrolase, EC 3.1.1.3) of *Mycoplasma gallisepticum* (Rottem & Razin, 1964), and a report on the lipase of *Staphylococcus aureus* (Crenshaw & San Clemente, 1964), purified bacterial lipases have not been fully characterized. Therefore, as part of this laboratory's interest in lipolytic microorganisms, an intensive study of the lipase of *Pseudomonas fragi* was undertaken, not only to enhance our immediate understanding of this enzyme, but also to provide a foundation for future work into its structure, mechanism of action, and biosynthesis. This paper reports the purification and partial characterization of the enzyme.

METHODS

Microorganism and enzyme. *Pseudomonas fragi* (NRRL B-25) was chosen for this investigation because it produces an extracellular lipase (Mencher, Ng & Alford,

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1965) and because a synthetic medium was available for production of the enzyme (Alford & Pierce, 1963).

Materials. The sources of chemicals and reagents were as follows: tributyrin (Baker grade) from J. T. Baker Chemical Company; crystallized human albumin from Nutritional Biochemicals Corporation; casein (purified) from Difco; Stractan from Stein Hall and Company; Astec 4135 from American Lecithin Company, Inc.; ammonium sulphate from Mallinkrodt Chemical Works; DEAE-Sephadex from Pharmacia; Silica gel G from Research Specialities Company; Chromosorb P from Wilkins Instrument and Research Inc.; chromatographic alumina F-20 from Alcoa Chemicals; disc electrophoresis reagents from Canalco; and methyl esters of fatty acids from the Hormel Institute. Synthetic triglycerides (98 % pure) were kindly supplied by Dr R. G. Jensen of the University of Connecticut, Storrs.

Assays. Lipase activity was assayed by titrimetric measurement of fatty acids released from lard (Alford & Pierce, 1963) or tributyrin, or by a pH-stat determination employing a titrigrath-titrator (Radiometer, Copenhagen). Emulsions were prepared by six passes through a hand homogenizer of 5 % (w/v) tributyrin in water with 0.5 % (w/v) Astec 4135 as the emulsifier. The respective assays are described in full in the appropriate figure legends. A unit of lipase is defined as that amount of enzyme which catalyzes the release of 1 μ mole of fatty acid from the substrate in 1 min. at pH 7.0 and 35°.

Protein was measured by the method of Lowry, Rosebrough, Farr & Randall (1951) with human serum albumin as the standard.

Proteinase activity was estimated by McDonald's modification of Anson's technique (McDonald, 1964) with casein as the substrate.

Disc electrophoresis. Disc electrophoresis was performed by the procedure of Ornstein (1964) and Davis (1964) in 7.5 % acrylamide gel with a constant current of 5 mA at 2–3°. Samples (200 μ g protein) were subjected to electrophoresis in duplicate; one of the gels was stained with Amido Schwartz and the other was sliced into 3 mm. sections which were pulverized and then assayed for lipase activity. In this way it was possible to relate the bands in the stained gel to lipase activity.

Sucrose density gradient centrifugation. Sucrose gradients were prepared in Beckman cellulose nitrate tubes, 2 \times 0.5 in., by layering 1.0 ml. 40 % (w/v) sucrose in 0.02 M-sodium phosphate buffer, pH 6.6, on the bottom, 3.0 ml. 25 % (w/v) sucrose next, and finally 1.0 ml. 10 % (w/v) sucrose on the top. Samples, generally 0.2–0.3 ml., in the phosphate buffer, were layered on the 10 % sucrose. Centrifugation was conducted in a Beckman Model L-2 ultracentrifuge with an SW 39L rotor at 124,000g for 17.5 hr at 2–3°. After centrifugation, tubes were pierced at the bottom with a hypodermic needle and 0.5 ml. fractions were collected.

Lipolysis of synthetic triglycerides. Lipolysis of triglycerides was carried out as previously described (Alford, Pierce & Suggs, 1964), except that Stractan rather than Astec was used to emulsify the substrate. Enzyme was added to the reaction mixtures at a concentration sufficient to catalyse the release of 10 mg. or less of fatty acids from the triglyceride in 2 hr at 35°. After incubation, reaction mixtures were extracted with petroleum ether, evaporated to dryness under a stream of nitrogen, and taken up in 1.0 ml. or less of chloroform for subsequent thin-layer chromatography.

Thin-layer chromatography (t.l.c.). Lipolysis products (free fatty acids, diglycerides, and monoglycerides) and unreacted triglycerides were separated by thin-layer chroma-

tography as described by Clément, Clément & Bezard (1962) on glass plates, 20 × 20 cm, coated with a 250 μ layer of silica gel G. Samples (5–10 mg., in chloroform) were applied as drops along the origin, 40 mm from the bottom of the plates. Chromatograms were developed with petroleum ether + ethyl ether + acetic acid (90 + 30 + 1 by volume). When the solvent front had migrated 130 mm, chromatography was terminated. Zones were detected with iodine vapour and then scraped from the plates for analysis by gas-liquid chromatography.

Gas-liquid chromatography (g.l.c.). To convert fatty acids to methyl esters for analysis by g.l.c., the method of Kates (1964) was used. To the sample, still adsorbed on silica gel, were added 4.0 ml. of 0.7 N-anhydrous methanolic hydrochloric acid. The mixture was refluxed for 2 hr at 80–100° and then cooled. One ml. water was added, and the mixtures were extracted three times with 3 ml. of petroleum ether. The extracted samples were evaporated to 1.0 ml. or less under a stream of nitrogen for analysis by g.l.c. with an F and M Model 700 gas chromatograph equipped with a flame ionization detector. Samples containing 5–25 μ g. of the fatty acid methyl esters were separated on Chromosorb P (45/60 mesh) coated with 20 % diethylene glycol succinate. The column temperature was 185°, and nitrogen was used as the carrier gas.

Production of crude lipase preparations. *Pseudomonas fragi* was grown in the synthetic medium of Alford & Pierce (1963) for 3 days at 20° under static conditions. The medium was separated from the cells by centrifugation and the supernatant culture fluid, which contained the lipolytic activity, was concentrated by ultrafiltration (Peterson & Sober, 1962). After concentration of the culture supernatant, insoluble material was removed by centrifuging and the supernatant fluid was dialysed against 20 vol. of distilled water for 3 hr and then lyophilized. The yield per litre of culture was 37 mg. of protein and 920 lipase units. The lyophilized material was stable for 4 to 5 months.

Preparation of DEAE-Sephadex for chromatography. DEAE-Sephadex (A-50, medium grade) was allowed to swell in an excess of water and, after fines had been removed by repeated decantations, was washed on a filter with 0.5 N-HCl, followed by water until the rinses were neutral. It was then washed with NaOH (0.5 N), followed by water until rinses were neutral. Next, the DEAE-Sephadex was washed with 0.02 M-NaH₂PO₄ until the pH of the rinse was 6.6, and then with 0.02 M-sodium phosphate buffer, pH 6.6. The anion exchanger was stored at 0–2° as a slurry in the phosphate buffer.

RESULTS

Purification of lipase

Purification was conducted at 0–2°. Crude, lyophilized lipase was dissolved in 0.02 N-sodium phosphate buffer, pH 6.6 (4 mg. protein/ml.) and solid ammonium sulphate was added with gentle stirring to 35% of saturation. The flask was allowed to stand for 15 min. and the material which precipitated was collected by centrifuging and dissolved in the phosphate buffer. The ammonium sulphate-fractionated material (3–5 mg. of protein in 3–4 ml. of buffer) was applied to a 1 × 20 cm. column of DEAE-Sephadex and eluted with buffer; 5 ml. fractions were collected and the flow rates varied from 20–30 ml./hr. After 30 ml. of eluent had passed through the column, a NaCl gradient was applied. The gradient was developed in a closed system with 2 M-NaCl in phosphate buffer in a reservoir leading to the mixing chamber which

contained 1 l. of buffer. In the first fractionations attempted, two peaks of proteolytic activity were eluted prior to elution of the lipase and some of this activity was detected in the lipase peak. To separate the lipolytic from the proteolytic activity, the NaCl gradient was interrupted at fraction 20, and at fraction 40, when all proteinase had eluted from the column, the gradient was reapplied and the lipase eluted. Fractions with high specific activity were pooled. The chromatographic separation is shown in Fig. 1. Table I showed that the lipase was purified 100-fold with an 18 % yield of activity.

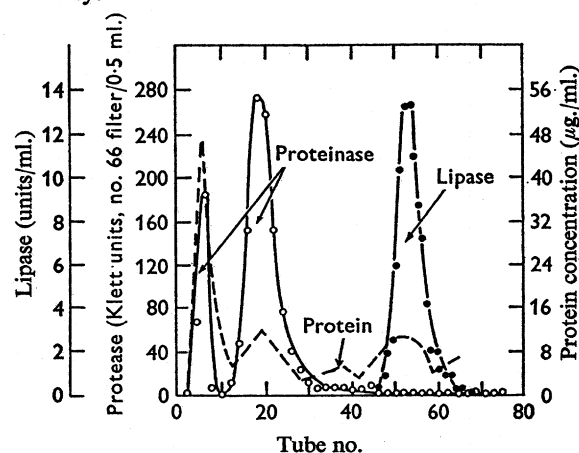


Fig. 1

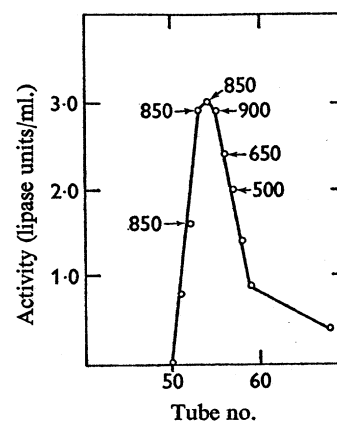


Fig. 2

Fig. 1. DEAE-Sephadex chromatography of *P. fragi* lipase. Chromatography was conducted as described in the text. O, Proteinase activity; ●, lipase activity; and ---, protein concentration.

Fig. 2. Specific activities of lipase in fractions from DEAE-Sephadex column. Conditions for chromatography as for Fig. 1, but peak taken from separate purification. Numbers with arrows designate specific activity.

Table I. Purification of *Pseudomonas fragi* lipase

	Total protein (mg.)	Total lipase activity (units*)	Yield (% of original activity)	Sp. act. (units/mg. protein)	Purification
Culture supernatant	320	2890	100	9	1
Ultrafiltration, dialysis, lyophilization	63	1568	54	25	3
(NH ₄) ₂ SO ₄ -fractionation	3.5	850	30	240	27
Chromatography on DEAE-Sephadex	0.55	510	18	925	103

* A unit of lipase is that amount of enzyme which liberated 1 μ mole of fatty acid from a 20% lard suspension in 1 min. at 35°.

Purity of lipase

Specific activity. The specific activities of fractions within the lipase peak suggested homogeneity in the leading portion of the peak with slight contamination in the tail portion (Fig. 2). Figures 1 and 2 represent two separate purifications.

Disc electrophoresis. Purification of the lipase was monitored by disc electrophoresis.

Prior to column chromatography lipolytic activity resided in two faint bands and the bulk of the protein moved with the free dye front in a band which was not lipolytic. However, DEAE-Sephadex chromatography completely eliminated the band at the dye front and the column purified preparation was comprised mainly of the two bands of

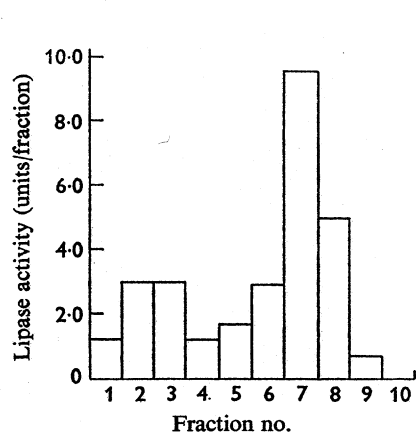


Fig. 3

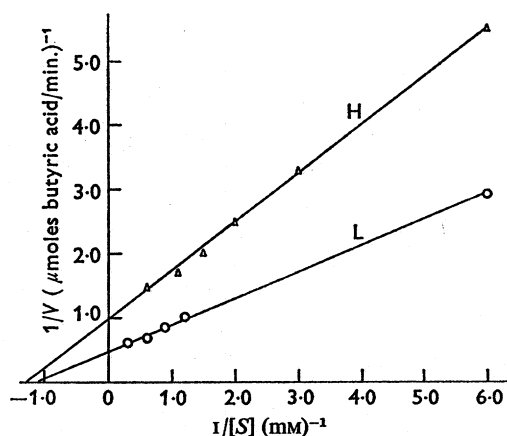


Fig. 4

Fig. 3. Sucrose density gradient centrifugation of ammonium sulphate-fractionated lipase. Procedures are described in the Methods Section. 'Heavy' material was obtained from fractions 2 and 3; 'light' from 6, 7 and 8.

Fig. 4. Double reciprocal plot for determination of K_m for lipases 'H' and 'L' with tributyrin. Initial velocity measurements were obtained with titrigrath-titrator by pH-stat determination (Desnuelle, Constantin & Baldy, 1955). Reaction vessels contained emulsified tributyrin at concentrations ranging from 0.16 to 3.30 mM, enzyme (constant: 'L', 21 μg. protein: 'H', 6 μg. protein) and H₂O to 10 ml. Assays were conducted at pH 7.2 and 25°. K_m was determined from the intercept with the x-axis.

Table 2. Disc electrophoresis of 'H' and 'L' activities

Distance from origin (mm.)	Lipase activity*		
	'H'	'L'	Purified lipase†
0-3	15.1	0.0	14.8
3-6	3.0	0.0	4.8
6-9	1.0	1.2	2.8
9-12	1.1	3.1	15.7
12-15	10.2	6.7	11.4
15-18	0.0	0.0	1.2
18-40	0.0	0.0	0.0

Disc electrophoresis of samples (200 μg. protein) was as described in Methods.

* Lipase activity expressed as ml. of 0.02 N base to neutralize the fatty acids released from lard substrate in 22 hr.

† Lipase purified by DEAE-Sephadex chromatography.

lipolytic activity. However, so little activity remained after electrophoresis that another method of separation was sought in order to ascertain whether these two components represented two lipases, two forms of the same enzyme, or an artifact of electrophoresis.

Density gradient centrifugation. Ammonium sulphate-fractionated lipase was

separated by sucrose density gradient centrifugation into a heavy component designated 'H' and a light component 'L' (Fig. 3). Ammonium sulphate-fractionated material was used rather than column purified lipase because of the excessive manipulations required to obtain sufficient quantities of column purified enzyme for the separation. Although the specific activities of the 'L' and 'H' components were about 235 and 250, respectively, as contrasted with that of about 900 for the column purified lipase, to avoid possible interconversion of the two components, no further purification was attempted. When these components were subjected to disc electrophoresis they were found to correspond to the two components formerly separated by electrophoresis (Table 2). Upon electrophoresis the 'H' component was resolved into two bands, one

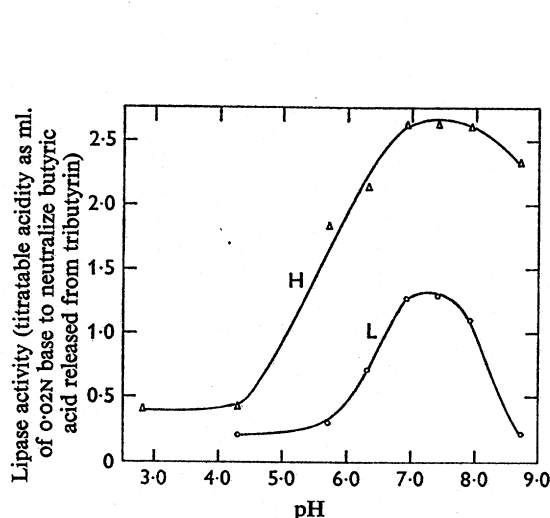


Fig. 5

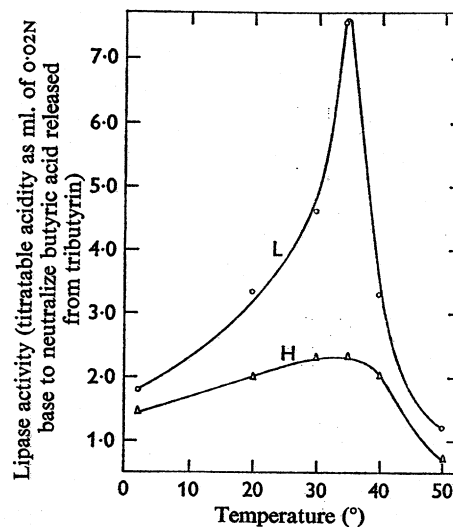


Fig. 6

Fig. 5. Effect of pH on activity of 'H' and 'L' lipases. Reaction mixtures contained 2 ml. of substrate (5%, w/v, tributyrin emulsion), 1 ml. of appropriate buffer (citrate-phosphate for pH 2.8, 4.3, 6.9; phosphate for pH 5.7, 6.3, 6.9, 7.4 and 7.9; tris for pH 8.7), 6 ml. of distilled H₂O, and 1 ml. of enzyme ('H', 4 μ g. protein; 'L', 3 μ g. protein). Mixtures were incubated at 35° for 20 min., 10 ml. of ethanol were added to them, and they were then titrated to pH 9.5 with 0.02 N-NaOH. Blanks containing water in lieu of enzyme were titrated and these values subtracted from those of the samples. The net values represent ml. of 0.02 N-NaOH required to neutralize the butyric acid released from the substrate.

Fig. 6. Effect of temperature on activity of 'H' and 'L' lipases. Lipase was assayed as described in legend for Fig. 5 at various temperatures at pH 7.0.

of which corresponded to that of the 'L' component, indicating that 'L' was present in the 'H' component. Further indication that the 'H' and 'L' activities represented two forms of the same enzyme was provided by the finding that they had similar *K_m* values for tributyrin. The values for the 'H' and 'L' forms were calculated to be 0.8 and 0.9 mM, respectively. Benzonana & Desnuelle (1965), using a Coulter counter, demonstrated that the significant parameter in the determination of *K_m* for insoluble substrates is interfacial area per unit volume. However, with the Coulter counter, the smallest particle that can be measured is 0.3 μ in diameter, and because 94 % by weight of the tributyrin emulsions used in our studies consisted of particles smaller than

that (as determined with the Coulter counter), determination of interfacial area with this apparatus was not possible. Nevertheless, repeated determinations of K_m in terms of molar concentration with different batches of substrate yielded the same value. Had the interfacial area per unit volume varied from one emulsion to the next, this would not have been the case. Thus, the molar concentrations here are an indirect representation of the interfacial area per unit volume and have been reported as such in this paper in the interest of comparison of the 'H', 'L', and column purified preparations. Double reciprocal plots for the determination of the K_m 's are shown in Fig. 4. It was also found that both components exhibited a 1,3-position specificity for the triglycerides tested (see below). Finally, an apparent range of pH 7-8 was optimal for both activities (Fig. 5), and the apparent temperature optima appeared to be the same, although the peak for the 'H' form was broad compared to that of the 'L' (Fig. 6).

Table 3. *Substrate specificity of purified Pseudomonas fragi lipase*

Hydrolysis of substrate was determined by pH-stat measurement with titrigraph-titrator at pH 7.0 and 25°. Reaction vessels contained substrate at concentration listed, purified enzyme (3 µg. protein), and H₂O to 10 ml. + Represents any activity above that of blank.

Substrate	Hydrolysis
Methyl butyrate: 90 mg./ml.	—
Methyl palmitate: 5 mg./ml.	—
Methyl oleate: 5 mg./ml.	—
Triacetin: 85 mg./ml.*	—
116 mg./ml.	+
Tripropionin: 2.5 mg./ml.	+
Tributyrin: 2.5 mg./ml.	+
Astec 4135: 2.5 mg./ml.	—
Lecithin (soy): 2.5 mg./ml.	—
Triglycerides of palmitic, oleic, and stearic acids	+
Diolein: 5 mg./ml.	+
Monolein: 5 mg./ml.	+

* Soluble in water at this concentration.

Characterization of purified lipase

Substrate specificity. The *Commission on Enzymes* (1965) has recommended that enzymes which hydrolyse insoluble glycerol esters of fatty acids be classified as lipases. To test the conformity of the purified *Pseudomonas fragi* lipase to this definition, the following substrates were tested: methyl butyrate, methyl palmitate, methyl oleate, Astec 4135, lecithin (soy), triacetin, tripropionin, diolein, and monolein (Table 3). To this list can be added the synthetic triglycerides of palmitic, stearic, and oleic acids used in the position specificity studies (see below). Only tri-, di-, and monoglycerides were hydrolysed by the lipase. Triacetin, which has limited solubility in water, was only hydrolysed at concentrations exceeding its solubility.

Position specificity for triglycerides. Crude preparations of *Pseudomonas fragi* exhibit a 1,3-position specificity for triglycerides (Alford, Pierce, & Suggs, 1964). In this respect the enzyme is similar to pancreatic lipase (Desnuelle & Savary, 1963). It was of interest to ascertain whether or not the bacterial enzyme would show a similar specificity after purification by DEAE-Sephadex chromatography or after density gradient fractionation. 2-Oleoyle palmito-stearin (POS), 1-palmitoyl diolein (POO),

2-palmitoyl diolein (OPO), and 2-stearyl-diolein (OSO) were hydrolysed by these enzyme preparations and the free fatty acids analysed. The specificity for the 1-3-position was still displayed by all three purified preparations (Table 4). That palmitic and stearic acids accounted for 6 and 7 % of the fatty acids released from OPO and OSO,

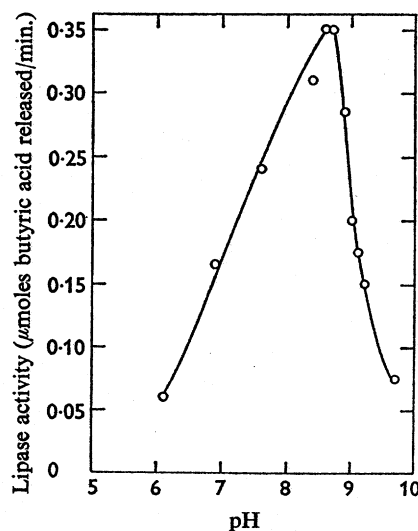


Fig. 7

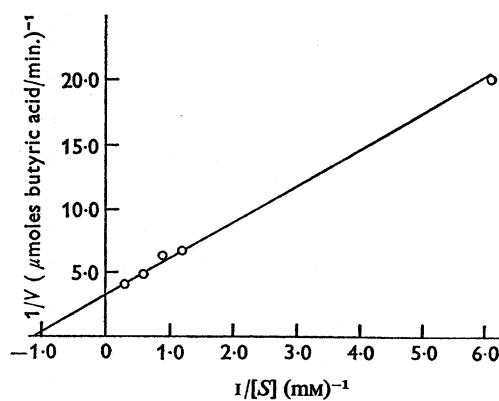


Fig. 8

Fig. 7. The pH optimum of purified *P. fragi* lipase. Initial velocity measurements were obtained with titrigrath-titrimeter by pH-stat determination. Reaction vessels contained 0.5 ml. of 5% tributyrin emulsion, 0.5 ml. purified lipase (6 μg. protein), and H₂O to 10 ml.

Fig. 8. Double reciprocal plot (1/v against 1/[S]) for determination of K_m of purified *P. fragi* lipase for tributyrin. Initial velocities were obtained as described in legend of Fig. 4. Reaction vessels contained emulsified tributyrin at varying concentrations (0.16–3.30 mM), 0.5 ml. of purified lipase (3.0 μg. protein), and distilled H₂O to 10 ml.

Table 4. Hydrolysis of synthetic triglycerides by 'H', 'L' and (purified) 'P' lipases

Triglyceride substrate	Weight of fatty acids released (%)								
	Oleic			Stearic			Palmitic		
	'P'	'H'	'L'	'P'	'H'	'L'	'P'	'H'	'L'
POS	2	3	2	49	49	50	49	47	48
POO	56	58	64	0	0	0	44	42	36
OPO	94	97	95	0	0	0	6	3	5
OSO	93	96	96	7	4	4	0	0	0

Enzyme was added in concentration sufficient to catalyse the release of 10 mg. or less of fatty acid from 50 mg. substrate in 2 hr. at 35° (approximately 2 units of enzyme based on assay with lard).

respectively, could be attributed to: (1) actual hydrolysis at the 2-position, (2) the presence of a small amount of triglyceride in which the fatty acid presumed to be in the 2-position was actually in the 1-position, (3) acyl migration, or (4) a combination of these possibilities.

pH optimum. To determine the pH optimum for the purified lipase, reaction mixtures were adjusted to the desired pH values with 0.01 N-HCl or NaOH and then assayed at those values with a pH-stat at 25°. An optimum of pH 8.6–8.7 with a rapid drop in activity above the optimum was found (Fig. 7).

Michaelis constant (K_m). The K_m for the purified lipase was found to be 0.9 mM with tributyrin substrate (Fig. 8).

Stability. Although crude preparations were relatively insensitive to freezing and thawing and to lyophilization, these procedures caused over 50 % inactivation of purified preparations. Further, the half-life of the enzyme decreased markedly with purification. Preparations with a specific activity of 700 displayed a half-life of 5 days at 2°, whereas those with a specific activity of 900 exhibited one of 15 hr.

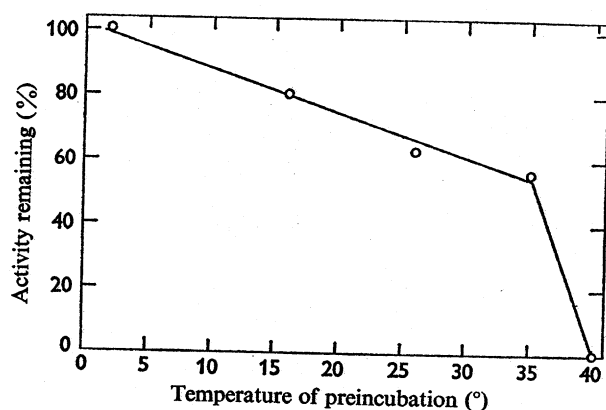


Fig. 9

Fig. 9. Effect of temperature on stability of purified *P. fragi* lipase. Samples of purified lipase (1 ml., 6 μ g. protein) were pre-incubated for 10 min. at temperatures ranging from 3° to 40°. Nine ml. of substrate-buffer mixture (prewarmed to assay temperature) were then added and the flasks assayed for lipase activity (Alford & Pierce, 1963).

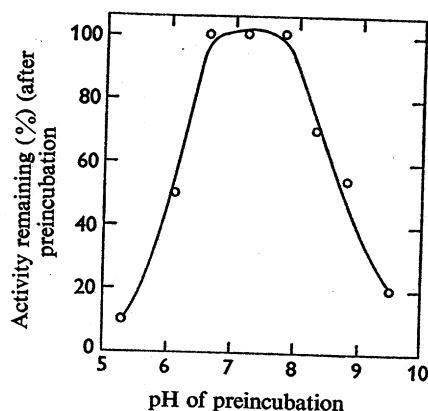


Fig. 10

Fig. 10. Effect of pH on stability of purified lipase. Samples of purified *P. fragi* lipase were adjusted to various values in the range pH 5.4–9.6, preincubated for 1 hr at 2° and then assayed at pH 7.0 and 35° (Alford & Pierce, 1963).

With regard to heat stability, the purified enzyme was completely inactivated in 10 min. at 40°, and a 50 % loss of activity occurred after 10 min. at 35° (Fig. 9).

To determine the effect of pH on stability of the purified lipase, the lipase was preincubated at various pH values for 1 hr at 2° and then assayed at pH 7.0 and 35°. Figure 10 indicates destruction of the enzyme below pH 6.6 and above pH 7.9.

DISCUSSION

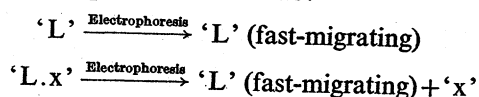
The borderline between true lipases and esterases is rather vague. Although the *Commission on Enzymes* (1965) has recommended that a lipase be termed a glycerol-ester hydrolase, reports of carboxylesterase activity in purified lipase preparations from porcine pancreas and fungi can be found in the literature (Desnuelle, 1961; Iwai, Tsujisaka & Fukumoto, 1964; Fukumoto, Iwai & Tsujisaka, 1964) and recently DeHaas, Sarda & Roger (1965) reported hydrolysis at the 1-position of phospholipids

by a highly purified preparation of porcine pancreatic lipase. While the possibility of contamination in the preparations cannot be ruled out altogether, it is quite possible that these enzymes represent lipases of a less specific nature and should be designated by a modified name. The lipase of *Pseudomonas fragi*, on the other hand, displays neither carboxylesterase nor phospholipase activity and thus it can be classified as a true glycerol-ester hydrolase. The requirement by this lipase for a glycerol moiety is of particular interest because it implies that the enzyme must bind to this portion of the substrate molecule in order to achieve a proper alignment of its catalytic groups, as suggested by Koshland (1963). That lecithin is not hydrolysed might be attributed to steric or electrostatic effects of the phosphorylcholine, preventing binding of the enzyme to the molecule or by preventing proper alignment of the catalytic groups. To test this possibility, purified lipase was preincubated with lecithin, and then triolein was added to the mixture. Hydrolysis of triolein proceeded at the same rate as when the enzyme had not been preincubated with lecithin, indicating that binding of lecithin by the enzyme had not occurred.

The pH optimum for activity of *Pseudomonas fragi* lipase has been reported to be pH 7.0 (Nashif & Nelson, 1953; Alford, Pierce & Sulzbacher, 1963). The present determination of pH 8.6–8.7 at first appears to be contradictory. However, these data can be reconciled by observing that the temperatures, substrates and methods used in the respective assays were different. The value of pH 8.6–8.7 was determined from initial velocity measurements, whereas that of pH 7.0 was not. Regarding the use of a pH-stat for determination of this optimum, Desnuelle, Constantin & Baldry (1955), using pancreatic lipase and olive oil, cautioned that such determinations are not valid because the fatty acids released from the substrate dissociate to a different degree depending upon the pH of the mixture. Thus, the observed reaction rate would actually reflect not only the rate of release of fatty acids from the substrate, but also the amount of dissociation of the acids. However, their statement is not applicable if $\text{pH} \gg \text{p}K_a$ for every species of acid produced. Furthermore, even this restriction is unnecessary if only a single acid is released. Knowing the $\text{p}K_a$, one can calculate the percentage ionization at each value from the Henderson-Hasselbalch equation and correct the initial velocity measurements accordingly. Such was the case with tributyrin.

The resolution of *Pseudomonas fragi* lipase preparations into two activities by electrophoresis and sucrose density gradient centrifugation, although not anticipated, is not strange. References in the literature to multi-molecular forms of proteins are commonplace and such techniques are often used to study these forms (Reithel, 1963). Regarding lipases specifically, Sarda, Maylié, Roger & Desnuelle (1964) reported the elution of a 'slow' and a 'fast' lipase peak during Sephadex chromatography of porcine pancreatic juice. The 'fast' lipase was converted to the 'slow' form by treatment with sodium deoxycholate and this led the investigators to speculate that the 'fast' component was a lipoprotein. Gelotte (1964) has reported the separation of pancreatic lipase into two enzymically active fractions by chromatography on Sephadex at various pH values and ionic strengths and has interpreted his data in terms of monomer and aggregate forms of the enzyme. Shahani & Chandan (1965) have used the analytical ultracentrifuge to study the association of milk lipase with other proteins. Depending upon the protein with which the enzyme was associated the activity was inhibited or enhanced by the formation of the complex. With respect to the lipase from *P. fragi*, the conclusion derived from the data in this investigation is that the enzyme exists in a

form which shall be designated 'L' for light and a heavy form 'L.x'. Disc electrophoresis of the two forms is pictured as follows:



and some undissociated 'L.x' (slow-migrating)

Pending structural analysis of the two forms, one can only speculate as to what 'x' is. The lipase could be complexed either with itself (dimerization), another protein, a lipid, or a carbohydrate. Conceivably, it could be complexed with some surface component of the cell. The enzyme was recently shown to be an extracellular one (Mencher *et al.* 1965), and although no significant cell-bound activity was detected in that study, this does not preclude association of the enzyme with the membrane or cell wall in an inactive form prior to its release into the medium. This possibility seems plausible in light of the report that the bound penicillinase of *Bacillus licheniformis* appears to be indistinguishable from the enzyme which is normally released into the medium, except for a difference in molecular weight (Pollock 1965). If the *Pseudomonas fragi* lipase is to be found associated with the cell wall or protoplast membrane in an inactive form, it would either have to be freed artificially in order to be identified by its activity, or be identified by immunochemical techniques or by structural analysis. The purified lipase should lend itself to such a study.

This work forms part of a dissertation submitted by J. R. Mencher to the Department of Microbiology and Tropical Medicine, Georgetown University, Washington, D.C., in partial fulfillment of the requirements for the degree of Doctor of Philosophy.

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